

Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'γ)₂ antibody

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We have developed a system for the targeted delivery of adeno-associated virus (AAV) vectors. Targeting is achieved via a bispecific F(ab)₂ antibody that mediates a novel interaction between the AAV vector and a specific cell surface receptor expressed on human megakaryocytes. Targeted AAV vectors were able to transduce megakaryocyte cell lines, DAMI and MO7e, which were nonpermissive for normal AAV infection, 70-fold above background and at levels equivalent to permissive K562 cells. Transduction was shown to occur through the specific interaction of the AAV vector-bispecific F(ab)₂ complex and cell-associated targeting receptor. Importantly, targeting appeared both selective and restrictive as the endogenous tropism of the AAV vector was significantly reduced. Binding and internalization through the alternative receptor did not alter subsequent steps (escape from endosomes, migration to nucleus, or uncoating) required to successfully transduce target cells. These results demonstrate that AAV vectors can be targeted to a specific cell population and that transduction can be achieved by circumventing the normal virus receptor.

Keywords: gene therapy vector, targeted transduction, parvovirus, adeno-associated virus (AAV), bispecific antibody

Recombinant adeno-associated virus (AAV) has emerged as a promising gene-delivery vehicle for human gene therapy. This small (20–25 nm) nonpathogenic human parvovirus is able to persist and express in both actively dividing and quiescent cells^{1–7}, does not provoke immune responses after vector transduction², and has a broad host range. One of the first *in vivo* studies using AAV vectors successfully delivered the CFTR gene to the lungs of rabbits and rhesus macaques⁸. These studies demonstrated vector transduction for up to 6 months and provided preclinical data that have facilitated a phase I toxicity trial in humans. More recent studies, mostly in rodents, have also demonstrated dose-dependent delivery and expression of various genes into a large variety of cells and tissues, including blood progenitor cells^{9,10}, lung⁷, muscle^{2,11}, and brain^{4,5}. To date, the data have been encouraging and have supported further development of this vector system for *in vivo* gene delivery.

AAV vectors carry only 145 nucleotides (comprising the viral inverted terminal repeats [ITRs] flanking the gene to be delivered) and lack all viral regulatory elements. This has allowed the use of tissue-specific transcriptional elements and enabled genetic targeting by means of tissue-specific gene expression^{12,13}. A potential improvement to this method of targeting would be the ability to physically target virion delivery. A number of other vector systems have been adapted, in an attempt to physically redirect vector tropism, with varying success^{14–20}.

Recently, we demonstrated rapid and selective uptake of AAV vectors after direct infusion in brain²¹. Although these data identified neurons as the primary target for AAV vector-mediated gene expression, not all neurons were permissive to infection, which suggested that the inability of vector to bind to specific cells could be a rate-limiting step for efficient gene delivery. Recent evidence suggests that AAV infection of human cells is mediated through attachment to cell membrane heparan sulfate proteoglycan²².

Although a requirement for heparan sulfate proteoglycan is currently being evaluated, its distribution among cell lines correlates with AAV infectivity, and soluble heparan sulfate can inhibit AAV infection²². Some cell lines that are naturally resistant to infection by recombinant or wild-type (wt) AAV^{23,24} were shown previously, and in this study, to have a reduced ability to bind virus. These include the human leukemic cell line UT-7/Epo (ref. 23) and the human megakaryocytic leukemia cell lines MB-02 and MO7e (ref. 24). The inability of AAV to bind to these cells is consistent with a reduced or absent cellular expression of AAV receptor. Based on this information, we chose these target cells as a model system for redirecting AAV vector infection. We showed that vector binding can be enhanced by targeting attachment to the megakaryocyte and platelet-specific α_{IIb}β₃ integrin (gpIIb/IIIa). Attachment is achieved by means of a bispecific (bs) F(ab'γ)₂ antibody in which one Fab'γ arm recognizes cell-surface α_{IIb}β₃ and the other recognizes the AAV capsid structure²⁵. Importantly, targeting does not appear to inhibit downstream steps required for productive infection (escape from endosomes, migration to the nucleus, or viral uncoating) by virtue of vector-mediated gene expression. In addition, we observed a decrease in the effectiveness of AAV vector-mediated gene delivery to permissive cells suggesting that the targeting approach was both specific and restrictive. Transduction of human megakaryocytic leukemia cell lines via this vector-bs F(ab'γ)₂ antibody complex is the first evidence of AAV targeting and suggests that this approach may be relevant for the transduction of other cell types resistant to natural infection.

Results

AAV vector transduction of hematopoietic cell lines. Although AAV vectors have reportedly transduced several cell types and tissues, the relative transduction efficiencies of many cell types are generally not

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known. Complicating these determinations is the observation that AAV vector transduction can be improved dramatically in some cells by adenovirus E4 open reading frame 6 gene expression, or by a number of physical or chemical manipulations²⁶⁻²⁸. Three cell lines have previously been shown to be nonpermissive for AAV infection. These are the human leukemia cell line UT-7/Epo (ref. 23) and the human megakaryocytic leukemia cell lines MB-02 and MO7e (ref. 24). Hematopoietic cells are clinically relevant targets for gene therapy of a large number of inherited, acquired, and infectious diseases. Even though several hematopoietic cell types have previously been shown to be transducible by AAV vectors, direct comparison of transducible and nontransducible lineages, and the block of efficient transduction have yet to be determined.

The relative transduction efficiency of DAMI and MO7e (human megakaryoblast cells) and K562 (human chronic myelogenous leukemia cells) was compared with the transduction efficiency of HeLa S3 carcinoma cells using a recombinant AAV vector (Fig. 1). The recombinant vector AAV β gal contained the β -galactosidase gene under control of the cytomegalovirus (CMV) enhancer/promoter. As demonstrated by β -galactosidase enzyme activity, there was essentially no AAV-mediated gene transfer to DAMI or MO7e cells. Alternative AAV vectors containing the β -galactosidase gene under control of a human snRNA promoter, or the human placental alkaline phosphatase reporter gene under control of a Rous sarcoma virus promoter, also failed to mediate transgene expression in these cells suggesting that the block was not at the transcription level (data not shown). K562 cells were transduced at a modest level, and HeLa S3 cells were readily transduced by the AAV β gal vector. When stained histochemically for β -galactosidase activity the transduction efficiency of both the DAMI and MO7e cells was less than 1×10^{-5} at a vector multiplicity of infection (m.o.i.) of 10 (data not shown). Adenovirus infection had little effect on AAV transduction of DAMI or MO7e cells, whereas transduction of HeLa S3 cells was increased considerably by subsequent adenovirus infection (Fig. 1). Adenovirus has been shown to overcome a leading-strand DNA synthesis block for efficient AAV

transduction of HeLa and HEK293 cells²⁶⁻²⁸ and was included here only to control for this possibility in the DAMI and MO7e cell lines. These findings suggest that the block for efficient transduction of the megakaryocyte cells is not at the level of AAV second-strand DNA synthesis, but possibly at an earlier step.

AAV vector binds poorly to human promegakaryocytic cell lines. To assess the role of virus attachment in transduction of these cells, the relative levels of specific AAV binding were determined (Fig. 2). HeLa S3 cells bound 10-fold more labeled AAV than did DAMI or MO7e cells. Furthermore, although over 90% of the HeLa S3 binding was inhibited by a 30-fold excess of unlabeled virus, the binding of AAV to DAMI, or MO7e cells was essentially unaffected by competing virus, suggesting a nonspecific interaction.

Expression of $\alpha_{IIb}\beta_3$ integrin complex. To address the issues of redirecting AAV vectors to a specific cell-surface target, we chose the well-characterized megakaryocyte-specific $\alpha_{IIb}\beta_3$ integrin. Expression of $\alpha_{IIb}\beta_3$ integrin was assessed by indirect immunofluorescence. DAMI and MO7e cells had high levels of $\alpha_{IIb}\beta_3$ integrin on the cell surface, whereas this receptor was not detected on the surface of K562 or HeLa S3 cells (Fig. 3).

Targeting AAV vectors with bispecific F(ab')₂ antibody. It was hypothesized that by redirecting vector binding to an alternative cell surface receptor (i.e., $\alpha_{IIb}\beta_3$ integrin present on DAMI and MO7e human megakaryoblast cells), the transduction efficiency of these cells could be increased. Bispecific A20AP2 F(ab')₂ was constructed to target AAV vector binding. The two monoclonal antibodies (Mabs) used to generate the bispecific molecule were AP-2 and A-20^{25,29,30}. Heterodimers were prepared by chemical crosslinking of Fab' arms—one with specificity toward $\alpha_{IIb}\beta_3$ integrin (AP-2 antibody [Ab]) and the other with specificity toward the intact AAV capsid (A-20 Ab)²⁵. The AP-2 Mab recognized the $\alpha_{IIb}\beta_3$ complex and did not react with its individual subunits. The major ligand for $\alpha_{IIb}\beta_3$ integrin is fibrinogen. As both fibrinogen and $\alpha_{IIb}\beta_3$ -specific Ab have previously been shown to be endocytosed via this receptor³¹, we hypothesized that AAV bound to $\alpha_{IIb}\beta_3$ may also be internalized in a receptor-mediated manner.

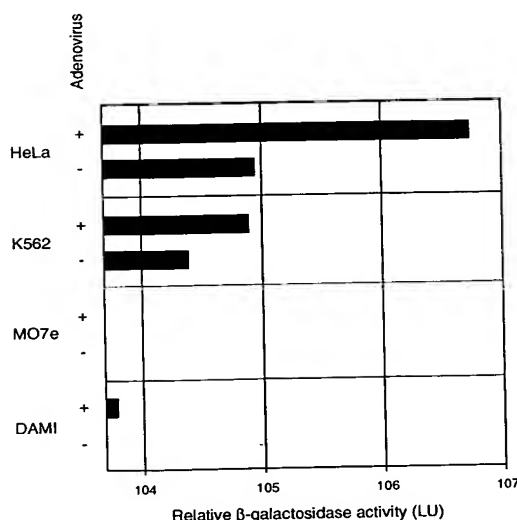


Figure 1. Quantification of β -galactosidase expression in cell lines transduced with recombinant AAV β gal vector. AAV β gal was incubated with cells for 1 h at 37°C. The indicated samples then received adenovirus (dl309) for 1 h. Activities of mock infections that received no vector were subtracted from each cell line. Results represent the average of duplicate samples.

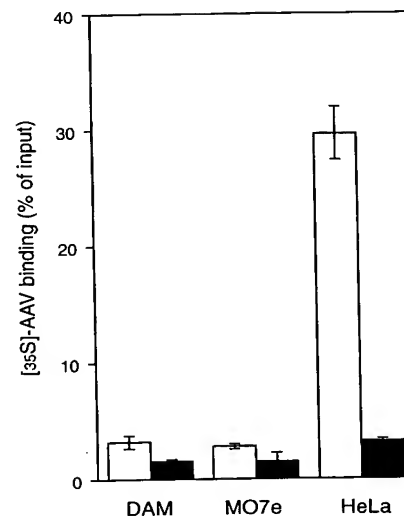


Figure 2. AAV binding to different cells. The indicated cells were incubated with [³⁵S]methionine-[³⁵S]cysteine-labeled virus for 1 h at 4°C and washed, and cell-associated activity was determined in a scintillation counter. Open columns (□) represent total binding. Closed columns (■) indicate binding in the presence of a 30-fold excess of unlabeled virus. Experiments were performed in triplicate and bars indicate the standard error of the mean.

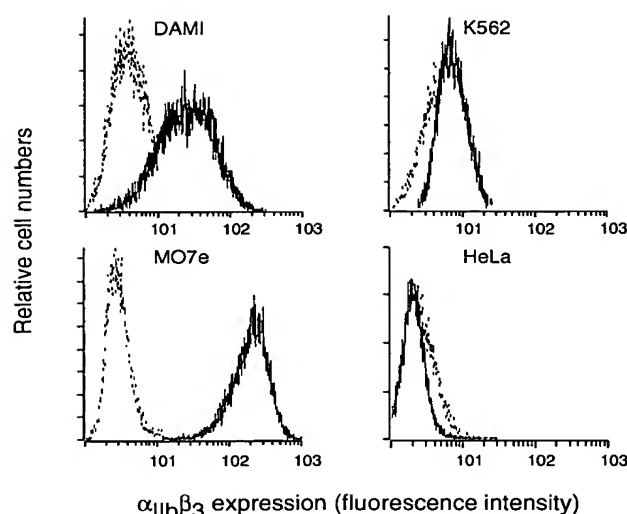


Figure 3. Fluorescence-activated cell sorting (FACS) analysis of $\alpha_{IIb}\beta_3$ integrin expression. Cells were treated with an excess amount of AP-2 Mab or control mouse IgG, washed and stained with phycoerythrin-conjugated polyclonal donkey antimouse F(ab) $_2$. FACS profiles of control IgG-stained cells (---), and AP-2-stained cells (—) are shown.

Studies were performed to determine whether bispecific A20AP2 F(ab' γ) $_2$ could mediate transduction of $\alpha_{IIb}\beta_3$ receptor-bearing cells. AAV β gal/bispecific Ab-mediated gene expression was determined in the presence or absence of competing $\alpha_{IIb}\beta_3$ Ab (AP-2) or noncompeting Ab (AK2), which recognizes platelet glycoprotein gpIb-alpha (Fig. 4). The AK2 Ab to gpIb-alpha (CD42b) recognizes the von Willebrand factor (vWF) receptor and the high-affinity thrombin receptor antigens expressed on cells of the megakaryocyte lineage. Bispecific A20AP2 F(ab' γ) $_2$ maximally increased AAV β gal-mediated β -galactosidase activity in $\alpha_{IIb}\beta_3$ -positive DAMI and MO7e cells approximately 70-fold above background (Fig. 4). Preincubation of these cells with Ab to $\alpha_{IIb}\beta_3$ (AP-2), but not Ab to gpIb-alpha (AK2), abrogated this effect (Fig. 4), demonstrating specificity of vector targeting. Transduction of $\alpha_{IIb}\beta_3$ -negative HeLa cells and K562 cells was decreased in the presence of bispecific Ab (Fig. 4). These results demonstrate that AAV vector, when complexed with the targeting bispecific A20AP2 F(ab' γ) $_2$, can specifically transduce a previously nonpermissive cell line. Furthermore, these results demonstrate that exclusive targeting of AAV vectors may be possible due to the decreased transduction efficiency of permissive cells in the presence of the bispecific Ab relative to the efficiency of the endogenous AAV receptor-mediated transduction of these cells.

To determine whether the increase in transduction mediated by the AAV β gal-bsAb complex reflected an increase in binding, binding of virus in the presence or absence of bispecific A20AP2 F(ab' γ) $_2$ was determined (Fig. 5). Radiolabeled AAV was preincubated with bispecific A20AP2 F(ab' γ) $_2$ then added to DAMI, MO7e, or HeLa cells in the presence or absence of competing AP-2 Ab. The bispecific A20AP2 F(ab' γ) $_2$ decreased AAV binding to HeLa cells approximately 10%, whereas AP-2 Ab alone had no effect on binding to these cell types (Fig. 5). AAV binding to DAMI and MO7e cells was significantly increased in the presence of bispecific A20AP2 F(ab' γ) $_2$, and could be effectively inhibited by pre-treating cells with AP-2 Ab (Fig. 5).

Human promegakaryocytic cells are permissive for infection by the bispecific Ab-AAV complex. Previously, it was shown that MO7e cells are nonpermissive for AAV infection. However, when

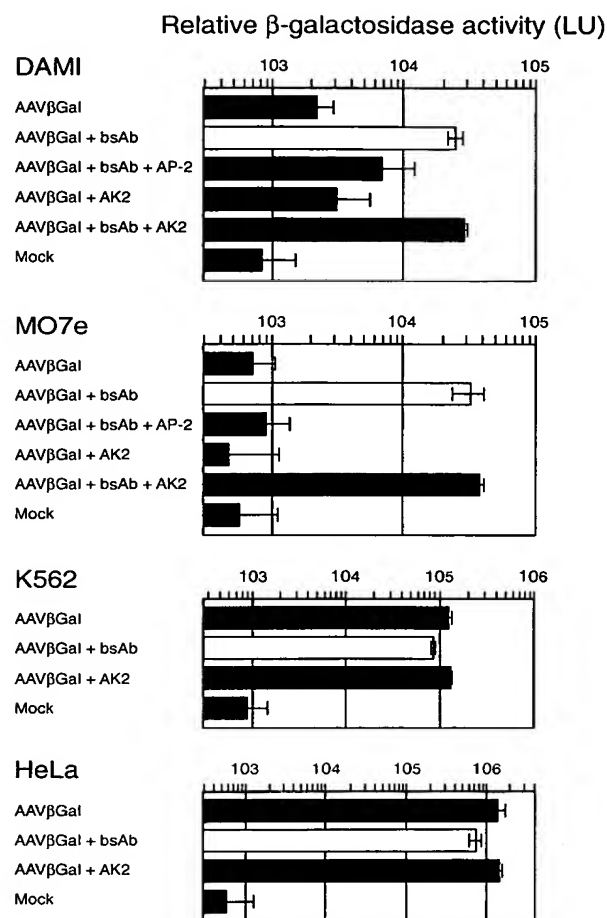


Figure 4. Targeted transduction of cells mediated by bispecific antibody. AAV β gal, or the AAV β gal-bispecific Ab complex was incubated with the various cells for 1 h at 37°C. Vector-bispecific antibody complexes were preformed for 1 h prior to addition to cells. When indicated, cells were pretreated for 1 h at 37°C with 25 ng/ml AP-2, or AK2 antibody. Following incubation with vector, the cells were washed and grown for 24 h at 37°C, and β -galactosidase activity was then determined as described in the Experimental Protocol. Activities of mock infections that received no vector are shown for each cell line. Results represent the average of triplicate samples from three to five experiments, and bars indicate the standard error of the mean.

AAV DNA molecules were physically introduced into adenovirus-infected MO7e cells, a productive infection took place supporting a likely block of virus entry 24 . We examined whether AAV redirected to $\alpha_{IIb}\beta_3$ integrin by A20AP2 bispecific Ab could undergo replication in these cells in the presence of helper virus. Equivalent numbers of HeLa S3, K562, MO7e, and DAMI cells were mock-infected, infected with AAV, or infected with the AAV-bsAb complex, in the presence or absence of 10 plaque forming units per cell of adenovirus. At various times postinfection samples were analyzed for AAV replication. HeLa and K562 cells demonstrated levels of AAV replication comparable to the levels of vector transduction seen in each of these cell lines. No viral replication was detected in either MO7e or DAMI cells (Fig. 6, panels 3 and 4). Although input signal persisted for several days as previously described 32 , we were unable to detect any increase in AAV genome copy number at any point postinfection in these cells.

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However, in the presence of bispecific A20AP2 F(ab')₂, AAV replication could be demonstrated in both the MO7e and DAMI cell lines (Fig. 6, panels 3 and 4). Replication of AAV in K562 cells was inhibited approximately 10-fold by bispecific A20AP2 F(ab')₂ consistent with a decrease in virus binding as demonstrated above (Fig. 5). Although replication was lower in MO7e and DAMI cells treated with bispecific Ab than in K562 cells, these data strongly support the conclusion that the targeted vector could facilitate a permissive infection by overcoming the block to virus uptake. Although K562 cells are known to be permissive to infection by recombinant AAV^{13,33,34}, the lower level of AAV replication in these

cells (relative to HeLa S3 cells); and lack of AAV replication initially in the megakaryocytic leukemia cell lines, could have been due to inefficient adenovirus infection. This possibility was discounted by reanalyzing samples from the replication assay for adenovirus-specific DNA replication. Adenovirus replication was confirmed in each of the cell lines (data not shown), suggesting that the absence of AAV replication was not caused by lack of adenovirus infection.

Discussion

Although AAV is known to have a very broad host-range, the efficiency of AAV vector-mediated gene delivery to different cell types varies greatly. Recently, it has been shown that AAV infection of human cells is mediated by heparan sulfate proteoglycan²² and that this receptor is absent on some cells of the erythroid-megakaryocytic lineage^{23,24}. We have confirmed that at least one mechanism responsible for the inefficient AAV transduction of these cells is the lack of this receptor to mediate virus binding and entry (Fig. 2). To overcome this obstacle, we have created a novel vector system composed of an AAV-bispecific Ab complex that is capable of binding to an integrin receptor expressed on these cells³¹. The vector-antibody complex is capable of specifically transducing human megakaryocytic leukemia cells that express this targeting receptor (Figs. 3 and 4). We observed a 90% reduction in AAV transduction of cells that did not express the targeting receptor. It remains to be determined whether this inhibition was due to steric hindrance by the bispecific Ab complex or another undertermined mechanism. Nonetheless, bispecific Ab is capable of targeting vector to a new cell surface receptor and at the same time inhibiting the interaction with the normal cellular receptor, resulting in both the selective and restrictive targeting of these vectors.

Specificity in the delivery of therapeutic genes will be important for effective gene therapy. For example, strategies for cystic fibrosis gene therapy rely on transduction of ciliated epithelial cells lining the airways of the lung. These cells are poorly permissive to vector infection³⁵. By targeting infection via an alternative receptor it may be possible to increase gene transfer to these cells. In this case, indiscriminate gene delivery to other cell types would probably not be harmful, but it would certainly diminish the effective gene dose to the target cell type. However, there are many other instances in which targeted delivery to a specific cell or tissue type is essential. A common approach to cancer gene therapy has been the insertion of a "suicide" gene into tumor cells *in situ*, with subsequent activation by prodrug therapy. The strategy here is that the transferred gene will confer susceptibility to the drug that will then kill the tumor cells. Transduction of non-tumor cells is to be avoided because these cells would also be killed by the therapy. For this reason, current suicide-gene approaches are only applicable for the treatment of well-defined solid tumors that can be physically targeted by injecting the gene vector directly into the tumor mass. If gene delivery to nontarget cells can be further reduced, targeted delivery of AAV vectors using the bispecific Ab system presented here could substantially limit secondary toxicity due to gene delivery to nontumor cells and may allow the treatment of poorly defined or even systemic tumors.

Although this work demonstrates a new principle and provides the groundwork for future studies aimed at redirecting AAV vector tropism, the most dramatic aspect of this study is the ability of the bispecific F(ab')₂ antibody to make a nonpermissive cell line permissive for AAV infection. Recent studies have confirmed that many target cells for therapeutic gene delivery are poorly permissive for vector transduction³⁵. The results presented here suggest that targeting strategies based on the use of bispecific antibody fragments may increase the

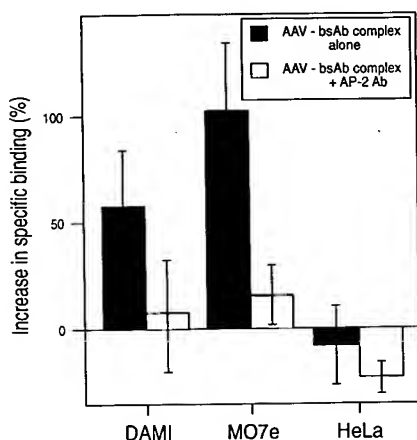


Figure 5. Binding of radiolabeled AAV to human megakaryocytic leukemia cell lines in the presence of bispecific F(ab')₂. Radiolabeled AAV was preincubated with bispecific A20AP2 F(ab')₂ then added to DAMI, MO7e, or HeLa cells in the presence (□) or absence (■) of competing AP-2 antibody. Cell-associated radioactivity, corrected for specific binding by subtracting values obtained in the presence of a 50-fold excess of unlabeled virus, was determined in a scintillation counter. Experiments were performed in triplicate, and bars indicate the standard error of the mean.

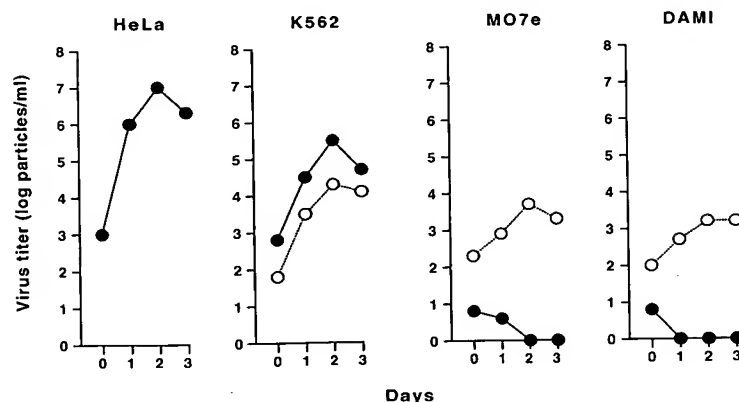


Figure 6. AAV infection of megakaryocyte cells mediated by bispecific antibody. Cells were incubated for 1 h at room temperature with AAV (●) or bispecific antibody-AAV complexes (○; 500 particles per cell), washed to remove unbound virus, infected with adenovirus, and incubated at 37°C for 1 h (0 days), 1 day, 2 days, or 3 days. Cells were lysed to release replicated viral genomes, and hybridization assays were performed as described in the Experimental Protocol. The figure shows the mean virus genome titer from duplicate cultures.

transduction efficiency of these cell types. For our studies, megakaryocytic leukemia cell lines were chosen as a model system with which to develop targeted AAV vectors. Although we were able to overcome the block to transduction of these cells, the level of vector-mediated gene expression in these cells is still quite low. Whereas bispecific Ab-targeted AAV vector transduction supports successful infection subsequent to binding and entry, the Ab may impede downstream steps such as the ability of the virus to translocate to the nucleus or to be uncoated. Further characterization of these steps may provide insight into potential improvements to this system.

Recent efforts have shown that both retrovirus and adenovirus vectors can be modified for targeting^{14–20}. We demonstrated that AAV can also be utilized for targeted delivery. In addition to demonstrating targeting capability, the simple virion structure, lack of toxicity after vector administration, and the potential for specific chromosome integrations as seen for wt AAV support further testing of this system. The use of a bispecific Ab to target vector delivery is attractive because it allows the use of the vast number of Mabs that have recently been developed and are already in use clinically^{36,37}. The ability to test additional Ab ligands with the hope of developing targeted AAV vectors for in vivo use should significantly advance the usefulness of this vector in human gene therapy.

Experimental protocol

Viruses and cell lines. DAMI, a human megakaryoblastoid cell line; HEK293, a human embryonic kidney cell line; K562, a human chronic myelogenous leukemia cell line; and HeLa S3, a human epithelial carcinoma cell line, were obtained from the American Type Culture Collection (Rockville, MD). HEK293 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY). DAMI cells were grown in Iscove's Dulbecco's modified Eagles medium (DMEM) containing 10% heat-inactivated horse serum (HS; Gibco). K562 cells were grown in RPMI-1640 media containing 10% FBS, and HeLa S3 cells were grown in modified essential medium (S-MEM, Gibco) containing 5% HS and 5% FBS. MO7e, human megakaryoblast cells, were obtained from Christopher Walsh (University of North Carolina, Chapel Hill, NC) and maintained in DMEM (Gibco) containing 20% FBS supplemented with recombinant interleukin-3 (8 U/ml). Adenovirus dl309 (ref. 38) has been described previously. wt AAV and recombinant AAV β gal virus were prepared from the plasmids pAB-11, pAAV/Ad, and psub201, which have been described previously^{39–41}. Briefly, pAB-11 contains the β -galactosidase gene under the control of the CMV immediate-early promoter/enhancer flanked by the AAV ITRs. Plasmid pAAV/Ad contains the AAV replication and capsid protein-coding sequences flanked by the Ad terminal repeats, and psub201 contains the entire AAV genome flanked by the AAV terminal repeats. Recombinant AAV was produced in adenovirus dl309 infected HEK293 cells and purified by two successive bandings on CsCl gradients, as described previously^{26,42}. Wt AAV was prepared in adenovirus-infected cells following transfection of psub201 plasmid DNA, as described previously⁴³. Purified virus was dialyzed into 10 mM Tris and 150 mM NaCl (pH 7.8) containing 10% glycerol and frozen at -80°C until required for use. Particle numbers for wt AAV were determined by protein quantitation (7.5 μ g protein equivalent to 10¹² particles). The titer of the recombinant virus, expressed in transducing units (tu), was determined by infecting HEK293 cells in the presence of adenovirus dl309 and staining at 36 h for β -galactosidase activity⁴⁴. Radiolabeled AAV was prepared by adding 50 μ Ci of [³⁵S]methionine-[³⁵S]cysteine (EXPRE³⁵S³⁵S, [³⁵S]Protein Labeling Mix; DuPont, Wilmington, DE) per ml to the medium 8 h after addition of adenovirus dl309 (m.o.i. = 5) to HEK293 cells transfected with psub201 DNA. Media was depleted of methionine for 2 h prior to addition of label. The infected cells were harvested at 48 h post infection and the virus was purified as described above. The activity of the labeled virus was approximately 5.5 \times 10⁻⁷ cpm per virus particle.

Antibodies. Three mouse Mabs were used in this investigation: A-20, which is an IgG antibody specific for intact AAV capsids²⁵; AP-2, which is an IgG antibody recognizing the integrin, $\alpha_{IIb}\beta_3$ (gplIbIIla; CD41) on human megakaryocytes and platelets^{29,30}; and AK2, which is an IgG1 antibody directed against the CD42b antigen (gplb-alpha) that serves as a receptor for vWF and as a high-affinity thrombin receptor. The hybridoma cell line producing the A-20 antibody was grown as an ascitic tumor in pristane-primed

(BALB/c \times CBA) F₁ mice. Ab AP-2 was provided as ascitic fluid. Antibody AK2 (Bioscience International, Kennebunk, ME) was obtained as protein A purified mouse ascitic fluid.

Preparation of IgG and F(ab')₂ fragments. IgG from mouse ascitic fluid was purified using immobilized protein A on a 1.0 ml AffinityPak Column (Pierce, Rockford, IL) and then desalted on a prepacked Exocellulose GF-5 Desalting Column (Pierce) according to the manufacturer's instructions. Ab was concentrated by ultrafiltration (Centricon-30; Amicon, Beverly, MA) and checked for purity using a size exclusion FPLC system fitted with a gel filtration column recommended for the size separation of biological macromolecules (Sephacryl S-200; Pharmacia, Uppsala, Sweden). F(ab')₂ fragments were prepared from the monoclonal IgG by digestion with immobilized pepsin. IgG at 1–2 mg/ml in 20 mM sodium acetate buffer, pH 4.5, was incubated at 37°C for 8 h with immobilized pepsin (Pierce). The solubilized F(ab')₂ and Fc fragments and undigested IgG were recovered from the immobilized pepsin gel. Undigested IgG, and Fc fragments capable of binding protein A were removed using a protein A column as described above. The F(ab')₂ samples were dialyzed overnight against phosphate-buffered saline (PBS), pH 7.4, to remove the remaining small Fc fragments (membrane molecular weight cutoff of 50 kDa) and concentrated to 0.5 ml by ultrafiltration. Fab' γ was obtained from F(ab')₂ by reduction in 20 mM 2-mercaptoethylamine (2-MEA) for 2 h at 21°C. 2-MEA was removed by dialysis against 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4, and the antibody fragments were again concentrated by ultrafiltration.

Construction of bispecific Ab. Heterodimeric bispecific F(ab')₂ A20AP2 was prepared by linking half-cysteine residues on A-20 and AP-2 Fab' fragments via thioether bonds with the use of the bifunctional crosslinking reagent, bismaleimido-hexane (BMH; Pierce). Fab' γ fragments from each antibody were prepared in 20 mM sodium phosphate buffer, 0.15 M NaCl, pH 7.4. A 1/20 volume of 25 mM BMH dissolved in dimethyl sulfoxide was added to one of the two mouse Fab' γ SH Abs. After 30 min at 21°C the maleimide-activated Fab' γ (Fab' γ_{mal}) was separated from other solutes in the reaction mixture by passage through a 5 ml desalting column (KwikSep Dextran; Pierce) equilibrated with sodium phosphate buffer, 0.15 M NaCl, pH 7.4. Fractions containing the maleimide-activated protein were pooled and concentrated by ultrafiltration. The Fab' γ_{mal} Ab was then added to the Fab' γ_{SH} Ab component of the heterodimer and incubated at 4°C for 18 h. Bovine serum albumin (BSA) was added to 0.1% and the reaction mixture was reduced with 2-ME at a final concentration of 40 mM for 45 min at 30°C and then alkylated with 25 mM iodoacetamide. The bispecific F(ab')₂ was separated from other products and residual reactants by passage through Sephacryl S-200 equilibrated in 0.2 M Tris-HCl, 10 mM EDTA, pH 8.0. Fractions containing the crosslinked F(ab')₂ were pooled and comprised approximately 77% of final preparation. The final reduction and alkylation step was designed to remove any untoward products, including F(ab')₂ homodimers, which may have formed by oxidation or disulfide exchange. The bispecific Ab would be expected to hold together during the reduction due to the presence of noncovalent interactions and nonreducible inter-Fab' bonds.

Transduction assays with recombinant AAV vectors. DAMI, MO7e, K562, or HeLa S3 cells (2 \times 10⁵ per well) were seeded onto 24-well plates 1 day prior to the experiments. In experiments to determine AAV-mediated β -galactosidase gene delivery to different cell types, AAV β gal vector (2 \times 10⁶ tu) was incubated with cells for 1 h at 37°C. Mock samples received no vector. Cells were then washed and incubated for 1 day at 37°C. The β -galactosidase activity of solubilized cells was measured in a luminometer using a chemiluminescent reporter assay (Gal-Light Plus; Tropix, Bedford, MA). In experiments comparing the transduction efficiencies of AAV β gal in the presence and absence of bispecific F(ab')₂ A20AP2 Ab, AAV β gal was preincubated with the indicated amounts of bispecific antibody in medium containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) for 1 h at room temperature (18–25°C). The Ab-virus complex was then added to cells (2 \times 10⁵, plated 1 day prior in 2 cm wells) and incubated for 1 h at room temperature. In experiments to determine adenovirus-mediated increases in AAV β gal transduction, cells were then washed and incubated with Ad dl309 at an m.o.i. of 2 for 1 h at 37°C. All cells were then washed and cultured for an additional day at 37°C when β -galactosidase activity was determined as described above. In similar experiments β -galactosidase transduction efficiencies were compared by staining the transduced cells using X-gal substrate⁴⁴.

AAV replication assays. In experiments measuring the ability of the indicated hematopoietic cell lines to support wt AAV infection and replication, cells (10⁵ cells/ml in 1.0 ml media) were incubated with 5 \times 10⁷ particles wt AAV and 10⁶ IU Ad dl309 at room temperature for 1 h then washed three

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times with media and grown at 37°C. Duplicate infections were performed in the presence or absence of 25 ng/ml bispecific A20AP2 F(ab')₂. Cells were harvested at the indicated times postinfection and low-M_r DNA was isolated as described by Hirt⁴⁵, analyzed by hybridization to a ³²P-labeled AAV-specific, or Ad-specific, DNA probe and quantitated by densitometric analysis.

Binding assays with wt AAV. In direct binding experiments measuring unconjugated AAV binding to different cell types, the indicated cells (10⁷ cells/ml in 0.4 ml media containing 0.5% BSA, 0.01% SDS, 20 mM HEPES, pH 7.4) were incubated with 10,000–50,000 counts per minute (c.p.m.) of [³⁵S]methionine-³⁵S-cysteine-labeled virus at 4°C for 1 h and then washed three times with PBS. Specific binding was determined by competing the binding of labeled virus with a 30-fold molar excess of unlabeled virus. In experiments comparing the effect of bispecific Ab on binding of AAV to different cell types, 40,000 c.p.m. of [³⁵S]methionine-³⁵S-cysteine-labeled AAV was preincubated with the indicated concentration of bispecific F(ab')₂ A20AP2 antibody for 1 h at room temperature and then added to 2 × 10⁵ cells and incubated at 4°C for 60 min followed by three washes with PBS. For all binding experiments, cells were solubilized in 200 µl of a 1% SDS solution in PBS and cell-associated activity was measured in a scintillation counter.

Immunofluorescence analysis of α_{IIb}β₃ distribution. The distribution of α_{IIb}β₃ integrins was assessed by indirect immunofluorescence using a fluorescence-activated cell sorter (FACSscan; Becton-Dickinson, Mountain View, CA). Cells at 2 × 10⁷/ml in PBS containing 0.1 mM Ca²⁺/Mg²⁺ and 2% BSA were first exposed to AP-2 Ab (1:1000 dilution of ascetic fluid) for 1 h at room temperature and then washed and treated for a similar period with phycoerythrin-conjugated donkey antimouse F(ab')₂ (Jackson ImmunoResearch, West Grove, PA) at 1:200 dilution. After a final wash to remove unbound reagents, the cells were examined in the presence of 1% paraformaldehyde fixative.

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